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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12Q 1/68, 1/70 — A1

(11) International Publication Number: WO 94/20640

(43) International Publication Date: 15 September 1994 (15.09.94)

(21) International Application Number: PCT/US94/02364

(22) International Filing Date: 4 March 1994 (04.03.94)

(30) Priority Data:
08/029,403 5 March 1993 (05.03.93) US

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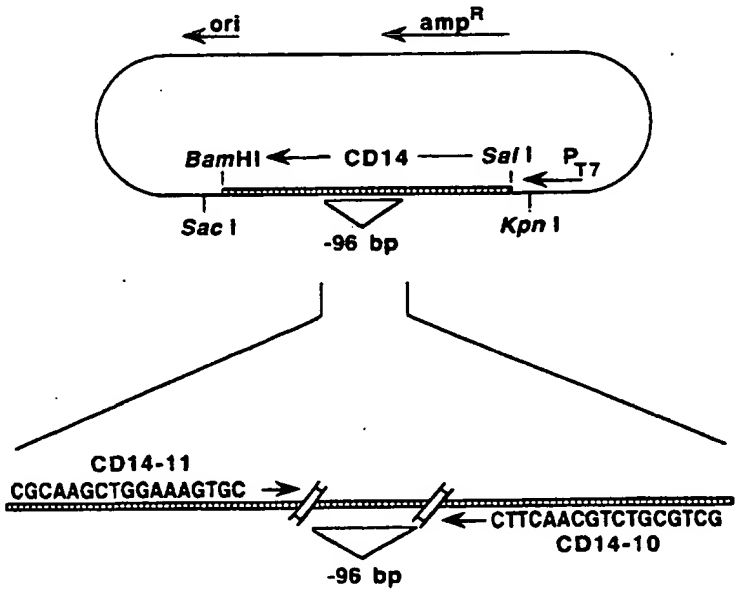
(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR HIV QUANTITATION



(57) Abstract

A method for quantitating nucleic acid in a test sample is provided. HIV is discussed as an exemplary nucleic acid, ranging from 100 to nearly 22,000,000 copies per milliliter of plasma (corresponding to 50 to 11,000,000 virions per milliliter). HIV was readily quantitated in HIV-infected patients. The method of the present invention is useful in assessing efficacy of antiretroviral agents, especially in early stage disease when conventional viral markers are often negative. Diagnostic reagents for quantitating HIV nucleic acid are also provided. Additionally a method for accurately determining cell copy number is provided.

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METHOD FOR HIV QUANTITATION

1. Field of the Invention

The present invention relates to a method for quantitating HIV nucleic acid in a sample and diagnostic reagents therefor.

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3. Background of the Invention

The progression of HIV-1 infection involves a variable clinical course with the development of AIDS generally occurring after seven to eleven years (Anderson, Munoz, Bacchetti). In the early stages of infection, the presence of HIV may be difficult to detect by circulating HIV p24 antigen, and levels of viral replication in peripheral blood appears to be quite low (Fauci, 1988; Fauci, 1991; Lane; Amadori; Pantaleo, 1991; Coombs; Ho; Saag; Clark).

Heretofore, polymerase chain reaction (PCR) methods have been developed to amplify low levels of HIV-1 RNA for monitoring low levels of viral replication associated with initial stages of HIV-1 infection. Previous PCR studies of HIV-1 RNA in plasma have generally been limited to qualitative (Ottmann, Murakawa) or semi-quantitative analyses (Holodniy, Aoki). In standard PCR methods, the absolute amount of product generated may not bear a consistent relationship to the amount of target sequence present at initiation of the reaction, particularly for clinical specimens (Noonan, Dickover). Both kinetics and efficiency of amplification of a target template are dependent on the starting abundance of the template and on the sequence match of the primers and target template, and may also be affected by inhibitors present in the specimen (Noonan, Dickover).

4. Summary of the Invention

The invention, in one aspect, provides a method for quantitating human immunodeficiency virus (HIV) in a sample by determining the copy number of a HIV nucleic acid target sequence in the sample. The method includes first adding to the sample, a known number of molecules of a competitive nucleic acid target sequence containing an upstream primer recognition region and a downstream primer recognition region, and extending between the upstream and downstream regions, a joining region whose properties permit a DNA molecule composed of the competitive target sequence to be electrophoretically separated from a DNA molecule composed of an HIV-1 target sequence including such upstream and downstream recognition regions.

If the HIV target and competitive target sequences are in single-stranded RNA form, corresponding complementary DNA sequences are formed. To the mixture are added upstream and downstream primers identified by SEQ ID NO:1 and SEQ ID NO:2, respectively, effective to bind to upstream and downstream recognition regions of the HIV and competitive target sequences in DNA form. These sequences correspond to highly conserved sequences in HIV-1, and include an inosine base at positions where base variation among HIV-1 strains has been observed. The sample is then subjected to a selected number of polymerization/amplification cycles in the presence of the primers, to produce amplified DNA products composed of the HIV and competitive target sequences from their DNA forms, amplified in proportion to the relative numbers of the competitive target and HIV-1 nucleic acid molecules originally present in step. The amplified DNA products formed are separated electrophoretically, and the relative amounts of said amplified DNA products are quantitated.

The method is preferably carried out with multiple samples containing different known numbers of competitive nucleic acid target sequence molecules and a constant unknown concentration of target sequence molecules. The above method may further include determining

the number of cells in a sample, from which the number of HIV target sequences/cell in the sample can be calculated, as described below.

In a more general aspect, the invention may be applied to quantitating an RNA or DNA virus in a virus-infected cell. The primers which are used in the method for amplifying viral target sequences and competitive target sequences are selected to correspond to highly conserved regions of the virus (as determined by viral strain sequence comparisons) and base positions in the primer regions at which variations occur are inosine bases in the primers, to reduce the differences in binding energies between the primers and different-strain viruses.

In another aspect, the invention includes diagnostic reagents for use in carrying out the method just described. The reagents include the competitive target sequence described above, and, if the HIV target and competitive target sequences are in single-stranded RNA form, reverse transcriptase reagents for use in producing corresponding cDNA molecules. Also included are the primers identified by SEQ ID NO:1 and SEQ ID NO:2, and polymerase chain reaction amplification reagents for use, in combination with the primers, in amplifying target and competitive DNA molecules in a sample.

In still another aspect, the invention includes a method for determining the cell number, micrograms DNA, or diploid genome equivalents in a cell sample by quantitating copy number of a single copy gene target sequence in the sample. The method includes first selecting a genomic target sequence in a human gene known to have two copies per diploid cell, and identifying upstream and downstream primer regions in the genomic target sequence.

To the cell sample containing the known genomic target sequence is added a known number of molecules of a competitive nucleic acid target sequence containing upstream and downstream sequences corresponding to upstream and downstream primer regions, respectively, and having properties which permit a double-stranded DNA molecule composed of the second competitive target sequence to be electrophoretically separated from a double-stranded DNA molecule composed of the genomic target sequence bounded by the same upstream and downstream recognition regions as the target sequence.

The target genomic sequence and added competitive sequence are then amplified by polymerase chain reaction (PCR) in the presence of primers corresponding to the upstream and downstream primer regions in the two sequences. The amplified DNA products are resolved electrophoretically, and the relative amounts of amplified double-stranded DNA products are determined. In one embodiment, the cell sample is derived from peripheral blood mononuclear cells, and the upstream and downstream primers have sequences: SEQ ID NO:3 and SEQ ID NO:4.

These and other objects and features of the invention will become more fully apparent when the following detailed description of the invention is read in conjunction with the accompanying drawings.

Brief Description of the Drawings

Figs. 1A-1D show the sequences of oligonucleotide primers used in the present invention: primer GAG04 (SEQ ID NO:1) (Fig. 1A), primer GAG06 (SEQ ID NO:2) (Fig. 1B), primer CD14-11 (SEQ ID NO:3) (Fig. 1C) and primer CD14-10 (SEQ ID NO:4) (Fig. 1D);

Fig. 2A shows a schematic diagram illustrating plasmids for wild-type HIV-I *gag* target sequence (pQP1) and deletion-containing competitive template (pQP1D80) and relative positions of amplification primers. Figs. 2B and 2C show HIV-1 RNA quantitation by the present method where PCR products derived from a transcript of the full length HIV target sequence (input per reaction = 2000 copies) (upper band, 260 bp) and from known amounts of a transcript from a competitive sequence (180 bp product) are electrophoretically separated and ethidium bromide stained (Fig. 2B). Further, where the log10 of the ratio of the signal for the competitive template-derived product over the signal for the wild-type target sequence-derived product (corrected for molar ratio) is plotted against the log10 of the copy number of added competitive template to determine the (unknown) copy number of the target sequence (Fig. 2C);

Figs. 3A and 3B are similar to Figs. 2B and 2C, respectively, but showing HIV-1 DNA quantitation rather than HIV-1 RNA quantitation;

Figs. 4A and 4B are similar to Figs. 2B and 2C, respectively, but where the sample tested is from an HIV-1 infected individual, rather than from a plasmid-derived HIV-1 transcript;

Fig. 5 is a schematic diagram of a plasmid containing a CD14 target sequence having a 96 base pair deletion in the center region of the genomic sequence;

Fig. 6 shows a comparison of diploid genome equivalents determined by the method of the present invention and by cell counts after serial dilution of a peripheral blood mononuclear cell (PMBC) sample; and

Figs. 7A-7C show longitudinal determinations of HIV-1 RNA copy number measured by the method of the invention (solid diamonds), p24 antigen levels (open circles), culturable virus (solid triangles), and CD4+ T cell count (open triangles) for three patients.

Detailed Description of the Invention

I. General Features of the Invention

The present invention includes a method for detecting and quantitating target nucleic acids present in a sample. In experiments performed in support of the present invention, quantitative competitive amplification analysis is used to determine RNA or DNA copy number by measuring relative amounts of coamplified polymerase chain reaction (PCR) products derived from a target sequence and a competitive target sequence. In the case of a DNA target a competitive DNA target sequence is used. In the case of an RNA target, a competitive RNA target sequence is used.

Typically, an RNA target and competitive RNA target sequences are converted to DNA molecules, using reverse transcriptase, as a first step in the amplification process. The DNA molecules contain the upstream and downstream primer recognition sequences which allow primer-specific amplification to generate DNA molecules by polymerase chain reaction. The DNA molecules are then used as template in amplification reactions.

The present invention improves the accuracy and precision of nucleic acid quantitation employing amplification reactions. Whereas other polymerase chain reaction quantitation methods use external controls to monitor levels of a target sequence, the present invention utilizes an internal control -- the competitive target sequence. The competitive target sequence contains upstream and downstream primer recognition regions that are functionally homologous to those of the target sequence (see below).

The method of the present invention allows the quantitation of an unknown amount of nucleic acid target sequence in a sample by addition of varying concentrations of a competitive target sequence to the sample before amplification. The relative amounts of resulting amplification products reflect the relative amounts of both the input competitive target sequences and unknown target sequences. Since the amount of the competitive target sequence is known, the unknown amount of the target sequence can be calculated.

II. Quantitative Competitive Amplification Analysis

A. Competitive Target Sequences

Target sequences are chosen that contain an upstream primer recognition region adjacent an internal nucleic sequence of a selected size followed by a downstream primer recognition region. The internal nucleic acid sequence is usually in the size range of 200-800 bases. Smaller and larger internal sequences can be used with the only requirement being the ability to separate the amplification products of a selected target sequence and a selected competitive target sequence, described below. Target sequences are typically selected that occur in regions of having conserved sequences between isolates. For example, viral nucleic acid sequences can

be compared and regions of homology identified between isolates. Primer recognition sequences are then chosen to take advantage of such regions of conserved homology. One such target region in an exemplary virus, HIV-1, is described below.

Primer recognition sequences need not have absolute homology between isolates. Sufficient homology should be present to allow the construction of primers that will selectively hybridize to the primer recognition sequences contained in the target sequences, *i.e.*, the sequences should be functionally homologous. The construction of functionally homologous primers is discussed below.

In the method of the present invention a competitive target sequence is selected to provide an internal amplification standard by which concentration of an endogenous target sequence of unknown concentration can be determined. The competitive target sequence has the following characteristics:

(i) it contains primer recognition sequences that are homologous to the primer recognition sequences in the target sequence; and

(ii) it is composed of a similar nucleic acid sequence as the target but modified so that after amplification the amplification product of the competitive target sequence has an altered phenotype relative to the amplified product of the target sequence.

As discussed above, sufficient homology should be present to allow the construction of primers that will selectively hybridize to the primer recognition sequences contained in the target sequences and the competitive target sequence.

The altered phenotype of the competitive target sequence is typically a size difference that can be detected subsequent to amplification by size fractionation, such as electrophoretic separation on an agarose gel or by capillary electrophoresis. One method of generating a competitive target sequence is as follows. The target sequence and primers complementary to the ends of the target sequence are selected. The target sequence is amplified by repeated rounds of primer extension (Mullis; Mullis, *et al.*). The amplified product is isolated. This isolated sequence can then be modified to generate a competitive target sequence by creating an internal deletion or insertion.

Alternatively, a restriction endonuclease cleavage site may be introduced into or deleted from the competitive target sequence, relative to the target sequence. Such modification of the competitive target sequence can be accomplished by standard methods (for example, site specific mutagenesis – Ausubel, *et al.*). In this method, the competitive target sequence and target sequence amplification products are subjected to restriction endonuclease digestion before size fractionation. For example, a restriction endonuclease cleavage site can be introduced into a competitive target sequence derived from HIV-1. To determine HIV copy number, coamplified HIV-1 target and competitive target PCR products are treated with a restriction endonuclease

that recognizes the restriction site. The restriction endonuclease-treated competitive target PCR products is completely cleaved by the restriction endonuclease to generate a shorter fragments. The restriction endonuclease digested amplification products are then resolved electrophoretically and relative amounts of the PCR products are quantitated.

Also, minor sequence differences present in the the competitive target sequence relative to the target sequence can be exploited to facilitate separation of the amplification products by, for example, analysis of the amplification products on denaturing gradient gels (Myers, *et al.*).

B. PCR Primers

The primers employed in the present invention are used in the amplification of a target sequence and a corresponding competitive target sequence (see below). Typically, both of these target sequences are in double-stranded DNA form. If RNA is being quantitated the RNA is initially transcribed to a cDNA. If DNA is being quantitated no manipulations are necessary prior to the polymerase chain reaction.

Primers and their primer recognition sequences are typically 15 to 40 nucleotides long. The 5'-ends of the primers can contain sequences not complementary to the primer recognition sequences in the target: for example, sequences containing specific restriction enzyme cleavage sites can be attached to the primers. The primers should be functionally homologous to the primer recognition sequences of the target sequences and the competitive target sequences.

"Functionally homologous" describes 5'-and 3', *i.e.*, upstream and downstream, primers from which equal amounts of polymerase chain reaction products are obtained when equal amounts of two or more target sequences and a competitive target sequence are present in a sample. Additionally, the internal nucleic acid sequence between the upstream and downstream primer recognition sequences of two or more target sequences and the competitive target sequence should not significantly affect the amplification product levels. Since the target sequence and the competitive target sequence bind primers with comparable efficiency and are amplified equally well, increasing concentrations of the competitive target sequence can be used to titrate an unknown amount of HIV nucleic acid sequence present in the sample.

Hybridization of the primers to extraneous non-target recognition sequences is typically minimized by altering primer length or composition (*e.g.*, increasing the length of the primer and its corresponding recognition sequence, or choosing a sequence having a G/C content of greater than 50%). The primers can be synthesized by standard methods or purchased from commercial sources.

The primers used to amplify the target and competitive target sequences do not have to have 100% homology to the primer recognition regions in the target sequences. Nor do the primer recognition regions of the target sequence and the competitive target sequence have to

be 100% homologous. As discussed above, both primers and primer recognition sequences should provide similar rates of product amplification (functional homology) when equal amounts of target and competitive target sequences are present in a reaction.

Additionally, the primers may be synthesized to contain inosine at base-pair mismatches that exist between the primer recognition sequence in target sequences and a competitive target sequence. The use of inosine at potential base pair mismatch sites between primer residues and upstream and downstream primer recognition region residues in sequence divergent HIV isolates minimizes negative interaction energies caused by base pair mismatching at such sites. Thus, the use of inosine maintains homologous and competitive priming efficiency with HIV strains having divergent target sequences.

In an exemplary embodiment of the present invention, primers are prepared that are complementary to 5'-and 3'-regions of a highly conserved region of the HIV *gag* gene. The primers GAG04 (Fig. 1A, SEQ ID NO:1) and GAG06 (Fig. 1B, SEQ ID NO;2) (Piatak). The primers were designed to amplify an internal fragment of approximately 260 bp from wild-type HIV-1 *gag* target sequences. Alternatively, other primers effective for binding to other highly conserved regions of the HIV target genome may be used.

C. Quantitative Competitive Amplification Analysis Protocol

A typical quantitative competitive amplification analysis involves the selection of a target sequence and the generation of a corresponding competitive target sequence. The competitive target sequence is typically the same type of nucleic acid as the target sequence, *i.e.*, DNA target sequence/DNA competitive target sequence, RNA target sequence/RNA competitive target sequence. The use of RNA competitive templates for RNA quantitative competitive amplification analysis provides a stringent internal control for both the reverse transcription and the amplification reactions. Known amounts of competitive target sequence are mixed with a source sample containing an unknown amount of target sequence. These mixtures of nucleic acids are then amplified (for example, by thermal cycling amplification reactions; Perkin Elmer, Norwalk CT) and analyzed to determine the amounts of amplification products present for the target sequence and the competitive target sequence.

Usually when quantitative competitive amplification analysis is performed with a sample whose concentration is unknown, a first titration is performed by adding increasing amounts of the competitive target sequence over a broad concentration range. After obtaining a rough estimate of the amount of target sequence in a sample, or when a rough estimate of the target concentration is known, a quantitative competitive amplification analysis experiment is performed over a narrow concentration range for precise quantitation. Most accurate results

are obtained when target and competitive sequences are amplified at nearly equivalent concentrations.

Both DNA and RNA can be quantitated by the method of the present invention. The method is exemplified below for quantitating HIV RNA levels and can be used to monitor different stages of HIV infection or to monitor the effect of drug therapy on progression of the disease.

In experiments performed in support of the present invention, quantitative competitive amplification analysis is used to determine HIV-1 RNA copy number by measuring relative amounts of coamplified PCR products derived from the HIV-1 RNA target sequence and a competitive RNA target sequence. First HIV-1 RNA target and competitive RNA target sequences are converted to double DNA molecules. The molecules contain the same upstream and downstream primer recognition sequences which allow primer-specific amplification of both DNA molecules by polymerase chain reaction. The relative amounts of PCR products reflect the relative amounts of both RNA target sequences present in a sample.

In one embodiment of the present invention, the competitive RNA target sequence contains an internal deletion which allows the DNA amplification products of both the target sequence and the competitive target sequence to be electrophoretically resolved. For example, the present invention has been used to quantitate HIV RNA in samples when the sample contains from approximately 100 to more than 20,000,000 copies per ml sample. Alternatively, the competitive RNA target sequence may contain an internal insertion which will permit amplification products to be resolved by size-fractionation. The method of the present invention can be used to quantitate HIV DNA copy number.

In another embodiment of the present invention, quantitation of the levels of target sequence and competitive target sequence may be performed by using multiple different primers and competitive target combinations for detecting several sequences at the same time. For example, primers specific for certain HIV isolates, and corresponding competitive target sequences, may be simultaneously used in screening a large number of individuals for HIV infection. Multiple primers can be used in the same or parallel amplification reactions.

Many types of samples may be the source of target sequences analyzed by the method of the present invention. For example, a sample may be from a body fluid, such as blood, spinal fluid, semen, saliva, effusions, pus, amniotic fluid, and urine. Alternately, a sample may be from a tissue sample derived from an individual or from cultured cells.

The following general protocols were used to carry out experiments performed in support of the present invention.

1. RNA Amplification Reaction Conditions. Polymerase chain reaction conditions and protocols were generally similar to those found in commercially available kits (Perkin-Elmer, Norwalk, CT). Typically, a test sample was divided into eight replicate aliquots and analyzed in the presence of increasing amounts of the competitive target sequences (0-50,000 copies per reaction). The initial reaction was typically performed in a total volume of 30 μl and contained 5 μl of test RNA (corresponding to 5% or less of the total specimen), 5 μl of competing RNA preparation or water, primers and 30 U of cloned Moloney virus reverse transcriptase (BRL, Gaithersburg, MD). One aliquot from each specimen was analyzed without reverse transcription and in the absence of competitive target sequences. Specific primers or random hexamer primers can be used. Random primer methods were carried out essential as per the manufacturer's instructions (Bethesda Research Laboratories, Gaithersburg MD; Boehringer Mannheim, Indianapolis IN).

When random hexamer primers were used, the reaction mixture was held for 10 minutes at room temperature to allow for partial extension and stabilization of random hexamer primers. Conversion of RNA into cDNA was allowed to continue for 30 minutes at 42°C. This reaction was then adjusted to contain primers and additional buffer in a total volume of 60 μl. Amplification was performed as described in Example 4. Typically, 45 reaction cycles were performed using the following protocol: 94°C for 1 minute, 50°C for 2 minutes, 72°C for one minute, followed by a final incubation at 5°C for 5 minutes (Piatak).

2. DNA Quantitative Competitive Amplification Analysis. DNA samples may be obtained by standard methods (Ausubel, *et al.*; Maniatis, *et al.*; Sambrook, *et al.*; Piatak). Typically, DNA samples were heated to 100 °C for 5 minutes to ensure denaturation of high molecular weight DNA in the initial PCR cycles. The competitive target sequence, for example, pQP1D80 (see below), was digested with a restriction endonuclease and heated to the same temperature to avoid the possibility of differential denaturation kinetics.

As described in Example 5, the following PCR procedure was employed for target and competitive target sequence amplification: *3 cycles* at 97°C for 1 minute; 55°C for 2 minutes; 72°C for 1 minute; 37 cycles at 94°C for 1 minute, 55°C for 2 minutes, 72°C for 1 minute, followed by 5°C for 5 minutes.

3. Amplified Product Resolution. In experiments performed in support of the present invention amplified products were resolved on the basis of size using electrophoresis. After amplification, approximately 7% of each reaction product mixture was separated by electrophoresis in composite 2% Synergel (Diversified Biotech, Newton Center,

MA)-1% agarose (FMC Bioproducts, Rockport, ME) gels. Gels were stained with ethidium bromide for visualization under UV illumination.

Fluorescence of both wild type and competitive target product bands was quantitated, as described, using a Lynx 4000 Molecular Biology Workstation, using matched custom software (Applied Imaging, Santa Clara, CA). The fluorescence associated with the 180-base pair product was corrected by multiplication by a factor of 260/180 to enable direct comparison of corrected fluorescence intensity of the 180-base pair band with the fluorescence of the 260-base pair band or a molar basis.

In another embodiment of the present invention, the amplification reactions can be carried out in the presence of a radioactive isotope. The amplification products are then size-fractionated and the relative amounts of radioactivity in the amplified target sequences and competitive target sequences are compared. The amplification products can be size-fractionated on gels, and directed counted in appropriate instrumentation.

D. Quantitative Competitive Amplification Analysis

1. Preparation of Control and Competitive Target RNA Sequences. Target sequence and competitive target transcripts were prepared to serve as control RNA and competitive RNA targets, respectively, in RNA quantitative competitive amplification analysis. As described above, a one target sequence for HIV nucleic acid quantitation is a region in the HIV-1 *gag* gene, which is highly conserved among viral isolates (Myers). To prepare target sequence and competitive target transcripts, plasmids containing the sequence (pQP1) and a sequence identical to pQP1, except for an 80-base pair internal deletion (pQP1-80), were prepared (Piatak). To prepare these plasmids (Fig. 2A) an approximately 1420-bp *Sac*I to *Bgl*II fragment of the *gag* gene was subcloned in Bluescript KS (Stratagene, La Jolla, CA). The HIV sequences of the two plasmids were placed under control of a T7 promoter to allow generation of the corresponding *in vitro* transcripts. *In vitro* RNA transcripts were prepared using commercially available T7 transcription kits.

2. PCR Analysis Validation. Transcripts derived from plasmids pQP1 and pQP1D80 were used to validate the present HIV-1 RNA quantitation method. In experiments performed in support of the present invention a known amount of pQP1 RNA transcript was cotranscribed and coamplified in the presence of increasing amounts of pQP1D80 RNA. Relative amounts of pQP1 and pQP1D80 PCR products were measured as described above.

Since both DNAs derived from wild type HIV and competition target sequences bind primers equally well, the pQP1D80 template concentration which results in an equal

amount of product derived from both templates reflects the concentration of pQP1 target sequence present in the sample (competition equivalence point).

Competition equivalence points were determined by interpolation on plots of the log of the calculated ratio of the signal for the pQP1D80 target RNA template derived product over the signal for the pQP1 HIV-1 target sequence derived product (corrected for molar ratio) versus the log of the copy number of added pQP1D80 competitive target RNA template. The $\log_{10}$ of the ratio of the corrected fluorescence intensity of the 180-base pair band over the fluorescence intensity of the 260 base pair band was plotted as a function of the $\log_{10}$ of the number of copies of the competitive target added. Graphically, this transformation yields a linear plot. At the equivalence point, the corrected fluorescence of the 180 bp band should equal the measured fluorescence of the 260 bp band, and their ratio should be equal to 1. Interpolation on the plot for a y-axis value of 0 gives the number of copies present in the test sample.

Fig. 2B shows a video image of an ethidium bromide stained gel derived from a quantitative competitive amplification analysis experiment where a sample containing plasmid pQP1 RNA was titrated with known amounts of plasmid pQP1D80 RNA. PCR products were derived from transcripts of plasmid pQP1 containing the full length target sequence (upper band, 260 bp) and from known amounts of transcripts derived from the competitive target sequences, plasmid (pQP1D80) containing an internal deletion (180 bp product). Lane 1 shows a sample where PCR amplification occurred without converting RNA transcript to cDNA. This was performed to rule out the presence of contaminating DNA in the samples. Lanes 2-8 show PCR products derived from the sample when either 0, 100, 500, 1,000, 5,000, 10,000, or 50,000 copies of competing pQP1D80 RNA transcript were added per reaction.

Fig. 2C shows a competition equivalence plot used to determine copy number of the HIV target sequence in a sample relative to known amounts of the competitive target sequences. The experimentally determined value was 2062 pQP1 plasmid copies/reaction. The estimated input number of target transcript was 2000 copies/reaction.

3. DNA Quantitative Competitive Amplification Analysis and Validation.

Figs. 3A and 3B show a method for HIV DNA quantitation by quantitative competitive amplification analysis. Fig 3A shows a video image of an ethidium bromide stained gel derived from a quantitative competitive amplification analysis experiment where a sample containing plasmid pQP1 was titrated with known amounts of plasmid pQP1D80. PCR products were derived from plasmid pQP1 containing the full length target sequence (upper band, 260 bp) and from known amounts of the competitive target sequences, plasmid pQP1D80 containing internally deleted competitive target sequences (180 bp product). Lanes 1 and 10 show

molecular weight standards. Lanes 2-9 show PCR products derived from the sample when either 0, 2, 10, 20, 100, 200, 1,000 or 5,000 copies of competing pQP1D80 were added per reaction.

Fig. 3B shows a competition equivalence plot used to determine copy number of the HIV DNA target sequence in a sample relative to known amounts of the competitive target sequence. The experimentally determined value was 10 pQP1 plasmid copies/reaction; the estimated input of target plasmid was 10 copies per reaction.

E. RNA Quantitative Competitive Amplification Analysis in Patient Plasma Samples

1. Patient Plasma Sample Preparation. All patients were evaluated at the University of Alabama at Birmingham where Human Subjects Review Board approval and informed consent were obtained. Blood specimens were collected in acid-citrate-dextrose (ACD) and processed within three hours of phlebotomy. Samples were centrifuged several times to ensure cell free specimens. Replicate aliquots of plasma were used immediately for virus culture or stored frozen at $\leq$ -70°C until the time of analysis.

2. RNA Preparation. Plasma samples were thawed. To increase the sensitivity and consistency of amplification of only virion-associated RNA, plasma was ultracentrifuged to pellet virus. Pellets were resuspended in a suitable buffer containing 1 mg/ml proteinase K and 0.1% SDS and incubated at 37°C for 1 hour to digest protein and replace RNA and then, repeatedly extracted with phenol:chloroform:isoamyl alcohol (24:24:1) followed by one extraction with chloroform. RNA was then precipitated with ethanol and pelleted by ultracentrifugation to maximize recovery. The RNA pellets were partially dried, then dissolved in 100 μl sterile, RNAse free water and stored at -70°C until subsequent analysis.

3. RNA Quantitative Competitive Amplification Analysis. HIV-1 virion-RNA enriched preparations were obtained from individuals for quantitation of HIV-1 RNA copy number. HIV-1 RNA was cotranscribed and coamplified in the presence of increasing amounts of transcript derived from pQP1D80. Relative amounts of HIV-1 and pQP1D80 PCR products were measured.

Since both DNAs derived from HIV-1 and pQP1D80 target sequences bind primers equally well, the pQP1D80 template concentration which results in an equal amount of product derived from both templates reflects the concentration of target sequence in the sample (competition equivalence point). For determining the competition equivalence point, the $\log_{10}$

of the ratio of the corrected fluorescence intensity of the 180-base pair band over the fluorescence intensity of the 260-base pair band was plotted as a function of the $\log_{10}$ of the number of copies of the competitive target sequences added. Graphically, this transformation yields a linear plot. At the equivalence point, the corrected fluorescence of the 180 bp band should equal the measured fluorescence of the 260 bp band, and their ratio should be equal to 1. Interpolation on the plot for a y-axis value of 0 gives the number of copies present in the test sample

To validate the quantitative competitive amplification analysis method for use in quantitating HIV-1 RNA, HIV-1 RNA in virus preparations was first quantified directly by electron microscopic particle counts (Layne, Bourinbaiar, McKeating) prior to quantitation by quantitative competitive amplification analysis.

Fig. 4A shows a video image of an ethidium bromide stained gel derived from an experiment where a plasma sample from patient BECH 0171, CDC Stage II was titrated with the transcript from pQP1D80. PCR products were derived from HIV RNA containing the full length target sequence (upper band, 260 bp) and from known amounts of transcript from the competitive target, plasmid pQP1D80 containing internally deleted competitive target sequences (180 bp product). Lane 1 shows a sample where PCR amplification occurred without converting RNA to cDNA. This was performed to rule out the presence of contaminating DNA in the samples. Lanes 2-8 show PCR products derived from the sample when either 0, 100, 500, 1,000, 5,000, 10,000, or 50,000 copies of competing pQP1D80 per reaction are added.

Fig. 4B shows a competition equivalence plot used to determine copy number of the HIV target sequence in the plasma sample relative to known amounts of the competitive target sequences. The experimentally determined value for HIV-1 RNA copy number was 700 copies/reaction or 13,800 copies/ml sample.

F. <u>HIV DNA Quantitation in Patient Samples</u>

For quantitative competitive amplification analysis total DNA was extracted preferably from the peripheral mononuclear blood cells (PMBC) or from lymph node tissue suspensions of HIV-infected individuals. To extract DNA, PMBC were centrifuged and washed prior to extracting DNA as described in Example 3. Total DNA, which includes HIV integrated in the cellular genome as well as any free nonintegrated viral DNA, was obtained.

Quantitative competitive amplification analysis assays were performed in the presence of the competitive target sequences pQP1D80 to determine HIV DNA copy number as described for determination of HIV RNA copy number, excluding the reverse transcriptase step.

In studies to determine the number of HIV DNA copies integrated into a cellular genome, an accurate determination of the number of cells present in a sample is necessary. Cell

number is usually determined by cell count which may be variable dependent on operator, cell viability and physical state of the cells or, alternatively, by performing serial dilutions of extracted DNA. The amount of DNA is then used to calculate the number of cells in the sample.

A more reliable method for calculating the number of cells in a sample has been developed based on quantitative competitive amplification analysis for normalizing HIV DNA copy number results to cell number. Typically, since HIV DNA integration is being investigated peripheral blood mononuclear cells were quantitated. The method involves determining the copy number of a single copy genomic gene, such as the CD14 gene present in PMBC. HIV copy number is normalized to CD14 copy number instead of normalizing to cell count number or micrograms DNA present in the sample. Alternatively, any single copy genomic gene in a given cell type can be used to determine cell number.

In experiments performed in support of the present invention a plasmid was constructed containing a region of the CD14 T cell lymphocyte gene with a 96 base pair deletion in pBluescript KS (pCD14D96) as illustrated in Fig. 5. Known amounts of pCD14D96 were then used as the competitive target sequence in DNA quantitative competitive amplification analysis titration reaction as described. The primers used in the amplification step were CD14-11 (SEQ ID NO:3) and CD14-10 (SEQ ID NO:4) illustrated in Figs. 1C and 1D, respectively.

Fig. 6 shows how the single copy genomic gene quantitative competitive amplification analysis quantitation method correlates with cellular quantitation (meticulously and exhaustively performed) by serial dilution of the cells. On the x-axis are plotted the CD14 gene diploid genome equivalents determined by serial dilution of the cell sample followed by cell count using a hemocytometer. The y-axis corresponds to the quantitative competitive amplification analysis determined equivalents of CD14/ diploid genome. As can be observed there is a linear correspondence between results obtained by both methods.

Table 1 compares PMBC quantitation by quantitative competitive amplification analysis and PMBC quantitation by serial dilutions of extracted PMBC DNA and absorbance measurements at 260 nm. Samples 1 and 2 are replicate specimens that contain 5×10^6 cells as determined by serial dilution of the DNA and corresponds to 10 million CD14 copies per diploid genome. When 5% of the sample is analyzed by quantitative competitive amplification analysis one would expect to detect 500,000 CD14 copies per reaction. The cell numbers obtained by quantitative competitive amplification analysis were close to that and corresponded to variations of either 3 or 1%. When lower cell copy number samples (500,000) were analyzed by quantitative competitive amplification analysis in two replicate sample, the variability of the method increased to only 4 and 19%. Therefore cell quantitative competitive amplification analysis, owing to its inherent sensitivity, appears to serve as a useful alternative

to other cell quantitation methods, especially at copy numbers too low to be measured by conventional methods or for tissue samples for which it is extremely difficult to dissociate cells suitably in order to obtain an accurate cell count and/or that avoids loss of cell material.

Table 1

Sample No.	(from serial dilution of DNA) Cell numbers (number of CD14 gene copies)	Number of CD14 Gene Copies per PCR Reaction	Number of CD14 Copies Determined by Quantitative Competitive Amplification Analysis	% of Variation
1	5×10^6 PMBC (10×10^6 copies)	500,000	515,714	+3%
2	5×10^6 PMBC (10×10^6 copies)	500,000	505,698	-1%
3	10^6 copies	50,000	47,806	-4%
4	10^6 copies	50,000	40,223	-19%

III. Comparison of HIV-1 RNA Quantitative Competitive Amplification Analysis Method with Other HIV Quantitation Methods

A. Patient Screening

Sixty-six consecutively enrolled HIV-1 infected subjects representing all stages of infection [CDC Stages I-IV] and 10 HIV-1 seronegative healthy donors were evaluated for virion-associated HIV-1 RNA by QC-PCR. Infected subjects were also tested for culturable virus and for p24 antigen using both standard and immune complex dissociation (ICD) test procedures. For example, culturable virus was determined in duplicate or quadruplicate, as described (Saag, Clark). Plasma sample were not filtered to eliminate inadvertent loss of virus. Regular and immune complex dissociated p24 antigen determinations were performed in duplicate using the Coulter Diagnostics kit assay (Coulter, Hialeah, FL), according to manufacturer's recommendations. For each subject, all virologic measurements were made using a single plasma sample, which was divided and frozen in replicate aliquots.

Virion associated RNA was detected and quantitated in plasma specimens from all 66 HIV-1 infected subjects (Table 2). RNA copy numbers ranged from 1.00×10^2 to 2.18×10^7 HIV-1 RNA copies per milliliter of plasma (corresponding to 0.50×10^2 to 1.09×10^7 virions/ml). Plasma RNA data was analyzed using the ANOVA Duncan's Multiple Range Test and Tukey's Studentized Range Test as calculated by the SAS statistical analysis software package (SAS Institute, Cary, NC). Positive signals for wild type HIV-1 target sequences were

not observed in specimens from any of 10 uninfected control subjects. Additionally, positive signal were not obtained when the reverse transcription step was omitted, for samples from any of the HIV-1 infected patients.

Table 2

Virologic and Clinical Summary for 66
Consecutively Studied HIV-1 Infected Patients

Patient ID	CD4 + cells (cells/mm3)	HIV RNA‡ (copies/ml)	HIV p24 Ag		Plasma¶ Culture (TCID/ml)
			ICD† (pg/ml)	Reg‡ (pg/ml)	
CDC State I*					
HOBR 0961	920	2,617,400	386	660	125
SUMA 0874	853	1,485,000	892	1,250	625
BORI 0637	817	2,398,600	218	258	1§
INME 0632	739	2,427,900	275	360	1,000
WEAU 0575	358	355,400	258	329	0■
FASH 1057	262	21,783,600	5,072	5,406	10,000
CDC Stage II/III					
RIPH 0179	1080	40,800	39	0	0
HIDO 1099	823	36,000	0	0	0
ATKA 0381	760	9,400	0	0	0
HOJU 0143	720	38,900	0	0	0
MAMA 0341	705	113,400	31	0	0
ADFR 0194	703	24,200	0	0	0
TIMI 0852[I]	678	100+	0	0	0
BECH 0171	678	13,800	8	7	0
MALE 0264	644	198,800	0	0	0
ROJO 0331	640	78,700	0	0	0
BELI 1233	627	4,400	0	0	0
HAJO 0940[I]	624	13,800	0	0	0
ADDI 0101	616	128,300	0	0	0
FOMA 0784[I]	521	34,400	0	0	0
JUJA 0156	458	586,100	0	0	5
WATH 0272[I]	350	67,200	0	0	0
BAMA 0037	323	91,000	154	53	0
WAJO 1286	281	46,100	183	35	0
BAST 0514[I]	270	9,400	0	0	0

Patient ID	CD4 + cells (cells/mm3)	HIV RNA* (copies/ml)	HIV p24 Ag		Plasma¶ Culture (TCID/ml)
			ICD† (pg/ml)	Reg‡ (pg/ml)	
STMI 0862	257	92,900	574	350	5
STBO 1287	231	8,600	0	0	0
SLMI 0843	197	84,900	0	0	0
CDC State IVC2					
ALFR 0229	615	91,800	0	0	0
DOBE 0859	533	114,500	0	0	0
CALI 0950[I]	456	40,800	35	5	0
ARLA 0846	435	49,100	0	0	0
WAOL 0263[I]	425	32,800	0	0	0
GADA 1162[I]	424	192,500	0	0	0
MCSE 0176	360	341,000	5,000	1,070	100
CHJI 0774	332	33,300	0	0	0
EDWI 0817	267	73,100	0	0	0
GRJO 0849	243	94,700	0	0	25
MIWI 1278	236	173,600	79	0	0
SMDO 0157[I]	141	2,200,000	740	284	3,125
HEMI 0562	117	223,000	123	101	125
DAJO 0508	109	36,900	361	120	0
SMST 1012	95	738,900	765	90	0
DUSE 1020	82	655,500	179	119	5
WHWI 1106	67	104,500	221	27	25
NOWR 1192	54	469,900	209	20	2,135
MIGE 1132[I]	29	191,800	0	0	0
DABE 0775	27	448,200	173	153	5
EMJA 0809	21	625,900	187	226	25
CLRA 0703	10	687,900	396	40	25
CDC Stage IVC1					
DEDA 0006	57	1,309,000	233	14	10,000
VAST 0101[I]	56	664,500	450	25	0
DODO 0116	50	1,667,000	357	385	100,000
SZHO 1173[I]	37	808,300	631	195	625
MILA 0284[I]	32	815,100	0	0	0
BIJA 0205	32	4,744,000	1,920	1,050	10,000
NAPH 0073	14	1,804,000	480	480	1,000

Patient ID	CD4 + cells (cells/mm3)	HIV RNA (copies/ml)	HIV p24 Ag		Plasma¶ Culture (TCID/ml)
			ICD† (pg/ml)	Reg‡ (pg/ml)	
TIMI 0018	14	3,445,000	330	390	100
WATI 0855	7	9,300,000	606	302	625
MCMI 0063	7	232,000	221	12	625
LENA 1029	<5	2,500,000	53	0	125
FARO 1042	<5	4,800,000	199	203	625
RUTH 1145	<5	424,800	230	143	125
YOAL 0522	<5	2,600,000	205	5	125
JOJI	<5	1,606,000	2,460	885	100,000

* CD4 + cells determined by flow cytometry, within six weeks of sampling for virologic assessments.

** HIV RNA copy number per milliliter of plasma, as determined by quantitative competitive amplification analysis.

† Immune Complex Dissociated HIV p24 antigen.

‡ HIV p24 antigen.

¶ Tissue Culture Infectious Doses of virus per milliliter of plasma.

Centers for Disease Control classification system for staging of HIV-1 infection (Morbid).

§ Three days earlier, plasma cultures were positive at 10 TCID/ml with HIV-1 RNA level of 1,350,600 copies/ml.

■ Eight days earlier, plasma cultures were positive at 1,000 TCID/ml with HIV-1 RNA level of 216,400 copies/ml (Fig. 7C).

⧗ AZT or ddI therapy at time of study.

\+ Extrapolated value.

QC-PCR determined HIV-1 RNA levels in plasma differed between clinical stages, with levels for CDC Stage II/III patients (asymptomatic/persistent lymphadenopathy) [mean 78,200 copies/ml (n=22)] significantly lower than levels for CDC Stage IVC2 (ARC) [mean 352,100 copies/ml (n=23); $p \leq 0.05$], and RNA levels for CDC Stage IVC2 patients were significantly lower than levels for CDC IVC1 (AIDS) patients [mean 2,448,000 copies/ml (n=15); $p \leq 0.05$]. CDC Stage IVC1 (AIDS) patients had circulating virus levels comparable to those observed during the peak of viral replication in Stage I (primary infection) patients [mean

5,178,000 copies/ml (n=6); difference not significant], implying that late stage disease is characterized by a nearly complete loss of immunological control of viral replication.

There was also a significant correlation between increasing QC-PCR determined HIV-1 RNA levels and decreasing absolute CD4+ T cell counts [Spearman correlation coefficient r= -0.765, $p < 0.0001$]. Spearman correlation coefficients were calculated by the SAS statistical analysis software package. A non-linear regression analysis of these data yielded the equation $\log_{10}$ RNA = 4.43 + 1.77 × exp(-0.0049 × CD4) with an R^2 value of 0.56 ($p < 0.0001$).

The threshold sensitivity for RNA QC-PCR analysis was determined to be 100 copies/reaction (Piatak). While positive signals can be detected for as few as 10-20 copies, results were less consistent below 100 copies/reaction and quantitation was less reliable. QC-PCR analysis was typically performed with 0.5 ml plasma samples. However, pelleting virus from increased volumes of plasma for samples containing low numbers of virions did allow for increased overall sensitivity of detection.

For example, for one p24 antigen negative, culture negative CDC Stage II patient (patient TIMI 0852, Table 2), initial QC-PCR analysis of a 0.5 ml specimen aliquot gave a negative result. However, analysis of virus pelleted from 2.8 ml of a replicate plasma sample allowed unequivocal detection of the low amount of virus present in this patient's plasma (100 copies/ml), while no HIV-1 RNA was seen with analysis of comparable volumes of plasma from HIV-1 seronegative controls.

As seen in Table 2, virus culture and standard p24 antigen assays were much less sensitive, with positive results in 4/20 and 5/20 subjects with CD4+ T cell counts > 500/mm^3, 6/18 and 7/18 subjects with CD4+ T cell counts of 200-500/mm^3, and in 22/28 and 24/28 subjects with CD4+ cells fewer than 200/mm^3, respectively. Circulating levels of plasma virus determined by QC-PCR also correlated with, but exceeded by an average of nearly 60,000-fold (Table 2) titers of infectious HIV-1 determined by quantitative endpoint dilution culture of identical plasma aliquots. For calculation of ratio of total virions determined by RNA quantitative competitive amplification analysis to culturable virus, specimens that did not contain culturable virus were assigned a TCID value of 1 to avoid 0 values. The ratios calculated therefore represent a lower estimate of true ratios of total circulating virions to culturable virus. Several virologic and immunologic factors already identified in HIV-1 infection, including neutralizing antibody (Weiss, Robert-Guroff, Nara), viral envelope shedding (Moore), deterioration of other viral components (Layne, Bourinbaiar), and genotypically defective virus (Li, Meyerhans), likely contribute to the differences in levels of circulating virus determined

by RNA QC-PCR and titers of culturable virus. However, the minimum requirements for establishment of productive infection of primary mononuclear cells are not known. If more than one intact viral particle is required to achieve productive infection of a host cell, this would exaggerate the discrepancy observed between viral titers in plasma determined by QC-PCR compared to endpoint dilution culture. For HIV-1 propagated *in vitro*, total virions have been reported to outnumber culturable infectious units by 10^4 to 10^7 (Layne), ratios similar to those that we observed in plasma.

In separate control studies, analysis of triplicate aliquots of plasma samples from six different patients, containing mean HIV RNA copy numbers of 6.7×10^4 to 1.0×10^6 per ml, gave a mean standard deviation of 22%. Analysis of replicate aliquots of the same HIV-1 RNA preparation on six different days gave a standard deviation of 15%. This reproducibility contrasts with variability of up to 600-1000% reported for non-competitive PCR procedures (Holodniy, Noonan, Gilliland, Becker-Andre).

Figs. 7A-7C show representative data for longitudinal determinations collected from three of six individuals followed over time, beginning at the time of presentation with acute HIV-1 infection (CDC Stage I) and continuing through the establishment of chronic infection (CDC Stage II to IVC2). Each patient presented signs and symptoms of primary infection, but lacked detectable HIV-1 reactive antibodies by screening ELISA or Western immunoblots. Each patient subsequently seroconverted with a full spectrum of HIV-1 specific antibodies by day 50 (Clark).

Virion associated HIV-1 RNA levels peaked between eight and twenty-three days after the onset of symptoms, reaching values between 3.55×10^5 and 2.18×10^7 copies/ml, (corresponding to 1.78×10^5 to 1.09×10^7 virions/ml). Culturable virus (10-10,000 TCID/ml) and p24 antigen (258-5,406 pg/ml) peaked at approximately the same time as virion RNA levels, declining rapidly thereafter in parallel with virion RNA levels. Within the first 100 days following onset of symptoms, plasma RNA levels fell by between 20 and 235-fold from peak levels, but in marked contrast to p24 antigen and culturable virus, remained continuously quantifiable for the duration of follow-up in all patients.

Among the six patients who presented symptomatic acute infection, there appeared to be associations between profiles of viral load in plasma over time, as determined by RNA QC-PCR, and trends in CD4+ T cell counts and clinical status. For example, patient SUMA (Fig. 7A) showed an initial peak of HIV-1 RNA (1.49×10^6 copies/ml) that dropped by nearly two logs within the first month of follow-up. Viral RNA reached a minimum (2.30×10^3

copies/ml) at 278 days and remained at or below 1.50×10^4 RNA copies/ml out to 473 days of follow-up. Plasma viral cultures and p24 assays remained negative following the initial peak. This patient maintained a normal CD4+ T cell count (952-1108/mm^3) and remained entirely asymptomatic over the period of follow-up. In contrast, patient FASH (Figure 7B) showed the highest initial virus peak (2.18×10^7 copies/ml), and the highest persistent levels of circulating virus, in the range of 3.26×10^5 to 6.32×105 copies/ml over 301 days of follow-up. The highest CD4+ T cell count measured in this patient was 282/mm^3, with a subsequent progressive fall to 128/mm^3 and clinical progression to CDC Stage IVC2, despite antiviral therapy. Although anecdotal, these observations suggest that higher levels of circulating virus, and failure to effectively control virus replication following initial infection, may be associated with a negative prognosis. However, in addition to quantitative viral load, other virologic or immunopathologic factors including a syncytium inducing viral phenotype likely contribute to variable rates of CD4+ T cell depletion and clinical outcome (Tersmette, Stein). In this regard it is of interest that patient WEAU (Figure 7C), who experienced a dramatic decline in CD4+ cells despite a peak level of viral RNA of only 3.55×10^5 copies/ml, was infected with a virus isolate that was markedly cytopathic upon *in vitro* culture (Clark).

Using different PCR techniques, other investigators have estimated levels of HIV-1 RNA in plasma ranging from 0 to 1×10^6 copies/ml (Holodniy, Noonan, Stieger, Menzo, Jurriaans, Scadden, Bagnarelli), generally one or more logs lower than we observed for patients with comparable stage disease. The methods employed by these investigators differed in various significant ways from those we describe here, including in some instances, the use of noncompetitive PCR techniques that do not provide the level of stringent internal control that is a central feature of QC-PCR, the use of different target regions and primer sets, or direct extraction of RNA from plasma without pelleting of virions by ultracentrifugation and other steps to maximize recovery of viral RNA (Piatak).

B. Demonstration of HIV-1 RNA Association with Virions

To demonstrate conclusively that the HIV-1 RNA quantified by QC-PCR was virion-associated, samples of HIV-1 containing culture supernatant and plasma from infected patients were fractionated by buoyant density centrifugation on continuous (20-60%) sucrose gradients. The HIV-1 RNA peaks corresponded precisely to the peaks of HIV-1 p24 antigen, both of which localized to fractions of the expected specific gravity for HIV-1 particles (Barre-Sinoussi, Popovic, Levy). For the banded virus, the ratio of virions (assuming two HIV-1 RNA

molecules per virion) to p24 antigen (in picograms) was approximately 10^4:1, in good agreement with estimates based on other biophysical studies of HIV-1 virions (25). Similar ratios were seen for virus pelleted from plasma (Table 1), with QC-PCR determined virus RNA levels for most subjects exceeding p24 levels (measured in pg) by the expected 3-4 logs.

IV. Monitoring Effect of HIV-1 Infection Treatment with Drugs Effective to Inhibit HIV-1 Growth

In other experiments performed in support of the present invention the impact of therapeutic intervention on viral load was measured by RNA QC-PCR. Specifically, HIV-1 RNA plasma levels from a limited number of patients, before and after initiation of treatment with AZT was investigated. As shown in Table 3, AZT treatment resulted in up to a 39-fold decrease in circulating virus as measured by RNA QC-PCR (patient JOJI 0070), with a significant treatment effect for the entire group of patients [$p < 0.0001$]. Despite differences between the absolute levels of virus measured by RNA QC-PCR and by culture methods, treatment-associated decreases in plasma HIV-1 RNA levels were paralleled by decreases in culturable virus, where measured.

Table 3

Patient ID	HIV RNA (copies/ml)	HIV p24 Ag ICD (pg/ml)	HIV p24 Ag Reg (pg/ml)	Plasma Culture (TCID/ml)	AZT	Time on Treatment (weeks)
MCSE 0776	341,000	5,000	1,070	100	-	17
	118,000	5,025	1,530	1	+	
NOWR 1192	469,900	209	20	3125	-	1
	82,400	41	0	ND	+	
EMJA 0809	625,900	187	226	25	-	6
	75,500	195	216	ND	+	
TIMI 0018	3,450,000	330	390	100	-	2
	235,000	35	35	0	+	
NAPH 0073	1,800,000	480	480	1,000	-	17
	182,200	320	320	100	+	
MCMI 0063	232,000	221	12	625	-	6
	134,300	160	7	ND	+	
JOJI 0080	1,606,000	2,460	885	100,000	-	20
	40,800	263	58	10	+	

Patient ID	HIV RNA (copies/ml)	HIV p24 Ag*		Plasma Culture (TCID/ml)	AZT	Time on Treatment (weeks)
		ICD (pg/ml)	Reg (pg/ml)			
SLMI 0843	84,900	0	0	0	-	Week 0†
	18,000	0	0	ND	+	Week 1
	33,500	0	0	ND	+	Week 2
	28,100	0	0	ND	+	Week 6
	72,700	0	0	0	-	Week 7
ARLA 0846	49,100	0	0	0	-	Week 0
	7,300	0	0	ND	+	Week 1
	6,500	0	0	ND	+	Week 2
	11,200	0	0	ND	+	Week 6
	58,400	0	0	0	-	Week 7
MIWI 1278	173,600	79	0	0	-	Week 0
	21,900	28	0	ND	+	Week 1
	10,900	24	0	ND	+	Week 2
	9,200	31	0	ND	+	Week 6
	136,300	47	0	25	-	Week 7

* Parameters as for Table 2.

† For kinetic analysis of viral load by RNA quantitative competitive amplification analysis over a six week AZT treatment period, patients were studied prior to initiation of treatment (week 0), after 1, 2, 6 weeks of treatment, and one week after temporary discontinuation of treatment (week 7).

As shown in Table 3 the HIV plasma levels from three previously untreated patients, who were given AZT for six weeks followed by a one week period off treatment, were also investigated. The observed rapid decreases in QC-PCR determined circulating virus levels upon initiation of treatment (week 1), and the rapid rebound of virus to pretreatment levels after discontinuation of treatment (week 7), reveal the dynamic nature and high levels of ongoing viral replication in these patients.

Two of these patients completely lacked detectable levels of other viral markers and could only be monitored by QC-PCR, while the third had detectable levels of p24 only after immune complex dissociation.

The following examples illustrate, but in no way are intended to limit the scope of the invention.

Materials and Methods

Synthetic oligonucleotides were prepared using commercially available automated oligonucleotide synthesizers. Alternatively, custom designed synthetic oligonucleotides may be purchased, for example, from Synthetic Genetics (San Diego, CA).

Restriction enzymes were obtained from Boehringer Mannheim (Indianapolis IN), Bethesda Research Laboratories (Gaithersburg MD) or New England Biolabs (Beverly MA) and were used as per the manufacturer's directions.

Standard molecular and cloning manipulations were carried out according to established procedures (Ausubel, *et al.*; Maniatis, *et al.*; Sambrook, *et al.*).

Example 1

Patient Samples

All patients were evaluated at the University of Alabama at Birmingham where Human Subjects Review Board approval and informed consent were obtained. Blood specimens were collected in acid-citrate-dextrose (ACD) and processed within three hours of phlebotomy. After centrifugation (200 × g for 15 minutes), plasma was collected and centrifuged again (1,000 × g for 15 minutes) to ensure cell free specimens. Replicate aliquots of plasma were used immediately for virus culture or stored frozen at ≤ -70°C until the time of other analyses.

Example 2

RNA Preparation

For extraction of virion associated RNA, plasma samples were thawed and subjected to ultracentrifugation (Beckman Type 70.1 rotor, 40,000 rpm, one hour) to pellet virions. Pellets were resuspended in 20 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, adjusted to contain 1 mg/ml proteinase K and 0.1% SDS and incubated at 37°C for one hour. After repeated extraction with phenol:chloroform:isoamyl alcohol (24:24:1) followed by one extraction with chloroform, samples were adjusted to contain approximately 40 μg/ml of glycogen (as carrier) and 10 ng/ml 7.5 Kb synthetic RNA (BRL, Bethesda, MD) (as carrier, and to normalize total RNA content in the reverse transcription and PCR steps). RNA was then precipitated with ethanol at -20°C for 48 hours and pelleted by ultracentrifugation to maximize recovery. The RNA pellets were partially dried, then dissolved in 100 μl sterile, RNAse free water. Samples were stored at -70°C until subsequent analysis. *Primers:* Primers GAG04 (SEQ ID NO:1) and GAG06 (SEQ ID NO:2) have been described and were designed to amplify an internal fragment of either 260 bp (from wild-type HIV-1 target sequences) or 180 bp (from

the pQP1D80 competitive target sequences). To maintain equivalent and competitive priming efficiency with divergent sequences, primers incorporated inosine residues at the few positions where divergence from the conserved consensus sequence.

Example 3

DNA Preparation

Total cellular DNA was extracted using the Elu-Quik™ Kit (Schleicher and Schuell, Keene, NH); the extraction protocol supplied with the kit was adapted to facilitate the handling of multiple samples. For quantitative competitive (QC)-PCR analysis of cellular DNA, cell pellets (0.5-2.0 × 10^7 cells) were lysed in approximately 350 μl guanidinium thiocyanate buffer, supplemented to contain 0.5% Sarkosyl™. DNA was adsorbed to glass beads, washed and eluted using 800 μl binding buffer, 250 μl glass beads, 3 × 1 ml washer buffer, 2 × 1 ml salt reduction buffer and 3 × 100 μl sterile water. The eluted samples were adjusted to 0.15 M potassium acetate (KOAc), pH 5.5, and ethanol precipitated. Pelleted DNA was then rinsed with 70% ethanol, partially dried and redissolved in 60 μl sterile water. Typical DNA yields were 5-8 μg/10^6 unstimulated peripheral blood mononuclear cells (PBMC).

Example 4

RNA QC-PCR Analysis

Two plasmids were prepared, one containing the target sequence (pQP1), and another containing the identical sequence, except for an 80 base pair internal deletion (pQP1D80), sufficient to allow the derived PCR products to be readily resolved by electrophoresis (Piatak). *In vitro* RNA transcripts were prepared with commercially available kits. Final preparations in water were determined to be essentially free of degradation products by Northern blot analysis and were quantified by measuring absorbance at 260 nm. Aliquots were stored at -70°C until needed. PCR reaction conditions and protocols were generally similar to those found in commercially available kits (Perkin-Elmer, Norwalk, CT). Each test sample was divided into eight replicate aliquots and analyzed in the presence of 0-50,000 copies per reaction of competitive target sequences. The initial reaction was performed in a total volume of 30 μl and contained 5 μl of test RNA (corresponding to 5% or less of the total specimen), 5 μl of competing RNA preparation or water, and 30 U of cloned Moloney virus reverse transcriptase (BRL, Bethesda, MD). One aliquot from each specimen was analyzed without reverse transcription and in the absence of competitive target sequences. After 10 minutes at room temperature to allow for partial extension and stabilization of random hexamer primers,

conversion of RNA into cDNA was allowed to continue for 30 minutes at 42°C. This reaction was then adjusted to contain primers and additional buffer in a total volume of 60 μl. Amplification was performed with 45 cycles using the following protocol: 94°C for 1 minute, 50°C for 2 minutes, 72°C for one minute, followed by a final incubation at 5°C for 5 minutes.

After amplification, approximately 7% of each reaction product mixture was separated by electrophoresis in composite 2% Synergel (Diversified Biotech, Newton Center, MA)-1% agarose (FMC Bioproducts, Rockport, ME) gels in 20 mM Tris Acetate, pH 7.8, 1 mM EDTA. Gels were stained with ethidium bromide for visualization under UV illumination. Quantitation of fluorescence of both wild type and competitive target sequences product bands was performed on a Lynx 4000 Molecular Biology Workstation, using matched custom software (Applied Imaging, Santa Clara, CA), as described (Piatak). Competition equivalence points were determined by interpolation on plots of the log of the ratio of signal for the competitive target sequences derived product over the signal for the wild type target sequence derived product (corrected for molar ratio) vs. the log of the copy number of added competitive target sequences.

Example 5

DNA Quantitative Competitive Amplification Analysis

Prior to setting up PCR, test DNA samples for analysis were heated to 100°C for 5 minutes to ensure complete denaturation of high molecular weight DNA in the initial PCR cycles. The pQP1Δ80 competing DNA was linearized with *Eco*RI and also heated to 100°C for 3-5 minutes to avoid the possibility of differential denaturation kinetics. Reactions conditions were based on the recommendations of the commercial reagent supplier (Perkin-Elmer, Norwalk, CT), except that each reaction was conducted in a total volume of 50 μl, using 0.5 U/reaction of *Taq* polymerase. A typical reaction contained 4 μl of appropriate competitive target sequences preparation (or water), 6 μl test sample (corresponding to 10% of the total amount of test sample) and 40 μl of reaction cocktail consisting of pooled buffer, deoxyribonucleoside triphosphates (dNTPs) and enzyme to give the appropriate final concentrations. Reactions were conducted in 96-well microplates using a programmable thermal controller (Model PTC-100; MJ Research, Watertown, MA) and the following PCR cycle program: *3 cycles*: 97°C for 1 minute; 55°C for 2 minutes; 72°C for 1 minute; 37 cycles: 94°C for 1 minute; 55°C for 2 minutes; 72°C for 1 minute; followed by 5°C for 5 minutes.

Although the invention has been described with respect to particular embodiments, it will be appreciated that various changes and modifications can be made without departing from the invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Lifson, Jeffrey D.
Piatak Jr., Michael
Luk, Ka-Chung

(ii) TITLE OF INVENTION: Method for HIV Quantitation

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Law Offices of Peter Dehlinger
(B) STREET: 350 Cambridge Avenue, Suite 300
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94306

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Fabian, Gary R.
(B) REGISTRATION NUMBER: 33,875
(C) REFERENCE/DOCKET NUMBER: 4600-0108

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (415) 324-0880
(B) TELEFAX: (415) 324-0960

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: GAG04, Figure 1A

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 4..5
(D) OTHER INFORMATION: /note= "where N is inosine"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 15..16
(D) OTHER INFORMATION: /note= "where N is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: GAG06, Figure 1B

(ix) FEATURE:
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(B) LOCATION: 3..4
(D) OTHER INFORMATION: /note= "where N is inosine"

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(B) LOCATION: 6..7
(D) OTHER INFORMATION: /note= "where N is inosine"

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(A) NAME/KEY: misc_feature
(B) LOCATION: 11..12
(D) OTHER INFORMATION: /note= "where N is inosine"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 17..18
(D) OTHER INFORMATION: /note= "where N is inosine"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: CD14-11, Figure 1C

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: CD14-10, Figure 1D

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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IT IS CLAIMED:

1. A method for quantitating human immunodeficiency virus (HIV) in a sample by determining the copy number of a HIV nucleic acid target sequence in the sample, comprising

(a) adding to the sample, a known number of molecules of a competitive nucleic acid target sequence containing an upstream primer recognition region and a downstream primer recognition region, and extending between the upstream and downstream regions, a joining region whose properties permit a DNA molecule composed of the competitive target sequence to be electrophoretically separated from a DNA molecule composed of an HIV-1 target sequence including such upstream and downstream recognition regions,

(b) if the HIV target and competitive target sequences are in single-stranded RNA form, forming complementary DNA target sequences,

(c) adding upstream and downstream primers identified by SEQ ID NO:1 and SEQ ID NO:2, respectively,

(d) subjecting the sample to a selected number of polymerization cycles in the presence of said primers, to produce amplified DNA products composed of said HIV and competitive target sequences, amplified in proportion to the relative numbers of the competitive target sequences and HIV-1 nucleic acid molecules present in step (a),

(e) resolving the amplified DNA products from step (d) electrophoretically, and

(f) quantitating the relative amounts of said amplified DNA products.

2. The method of claim 1, wherein the HIV and competitive target sequences in step (a) are in single-stranded RNA form, and said forming includes treating the target sequences with reverse transcriptase in the presence of primers and nucleoside triphosphates.

3. The method of claim 1, wherein the HIV and competitive target sequence in step (a) are in double- stranded DNA form, and step (b) is omitted.

4. The method of claim 1, wherein one of the two target sequences, in double-stranded DNA form, has a unique restriction site, and said separating includes treating the amplified double-stranded DNA products from step (d) with a restriction endonuclease effective to cut double-stranded DNA at said unique site and separating the double-stranded DNA molecules electrophoretically.

5. The method of claim 1, wherein the amplified DNA products formed in step (d) have different numbers of basepairs, allowing the products to be separated electrophoretically.

6. The method of claim 1, wherein steps (a) through (f) are carried out in multiple samples containing different known numbers of competitive nucleic acid target sequences.

7. The method of claim 1, which also includes determining the number of cells in said sample, from which the number of HIV target sequences/cell in the sample can be calculated, said determining including the steps:

(a') selecting a genomic target sequence in a human gene known to have two copies per diploid cell,

(b') identifying upstream and downstream primer regions in the genomic target sequence,

(c') adding to the sample, a known number of molecules of a second competitive nucleic acid target sequence containing upstream and downstream sequences corresponding to said upstream and downstream primer regions in the genomic target sequence, respectively, and having properties which permit a DNA molecule composed of the second competitive target sequence to be electrophoretically separated from a DNA molecule composed of the genomic target sequence bounded by the same upstream and downstream recognition regions as the second competitive target sequence,

(d') adding upstream and downstream primers effective to bind to upstream and downstream recognition regions of the genomic and second competitive target sequences in DNA form,

(e') subjecting the sample to a selected number of polymerization cycles in the presence of the primers added by (d'), to produce amplified DNA products composed of said genomic and second competitive target sequences from their respective DNA forms, amplified in proportion to the relative numbers of the genomic and second competitive target nucleic acid molecules present in step (c'),

(f') resolving the amplified DNA products from step (e') electrophoretically,

(g') quantitating the relative amounts of said amplified DNA products, and

(h') correlating genomic target sequence amount to cell number in the sample.

8. The method of claim 7, wherein the genomic target sequence is contained within the human CD14 gene, for use in quantitating HIV DNA copy number in peripheral blood mononuclear cells.

9. The method of claim 7, wherein the upstream and downstream primers are SEQ ID NO:3 and SEQ ID NO:4, respectively.

10. Diagnostic reagents for use in quantitating human immunodificiency virus (HIV) in a sample by determining the copy number of an HIV nucleic acid target sequence in the sample, comprising

(a) a competitive target reagent containing a known number of a competitive nucleic acid target molecule, said molecules containing an upstream primer recognition region and a downstream primer recognition region, and extending between the upstream and downstream regions, a joining region whose properties permit a DNA molecule composed of the competitive target sequence to be electrophoretically separated from a DNA molecule composed of an HIV-1 target sequence including such upstream and downstream recognition regions,

(b) if the HIV target and competitive target sequences are in single-stranded RNA form, reverse transcriptase reagents for use in producing complementary DNA molecules from the RNA molecules present in the sample and in said RNA reagent,

(c) upstream and downstream primers identified by SEQ ID NO:1 and SEQ ID NO:2, and

(d) nucleic acid amplification reagents for use, in combination with said primers, in amplifying said DNA molecules composed of said HIV and competitive target sequences.

11. The diagnostic reagents of claim 10, wherein the competitive target molecule is a molecule whose DNA molecule differs in length from the DNA molecule derived from the HIV target sequence.

CATICTATTTGTTCITGAAGGGTACTAG Fig. 1A

GCITTIAGCCIGAAGTIATACCCATG Fig. 1B

CGCAAGCTGGAAAGTGC Fig. 1C

CTTCAACGTCTGCGTCG Fig. 1D

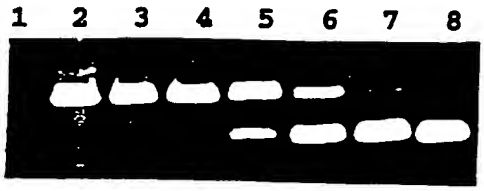

Fig. 2B

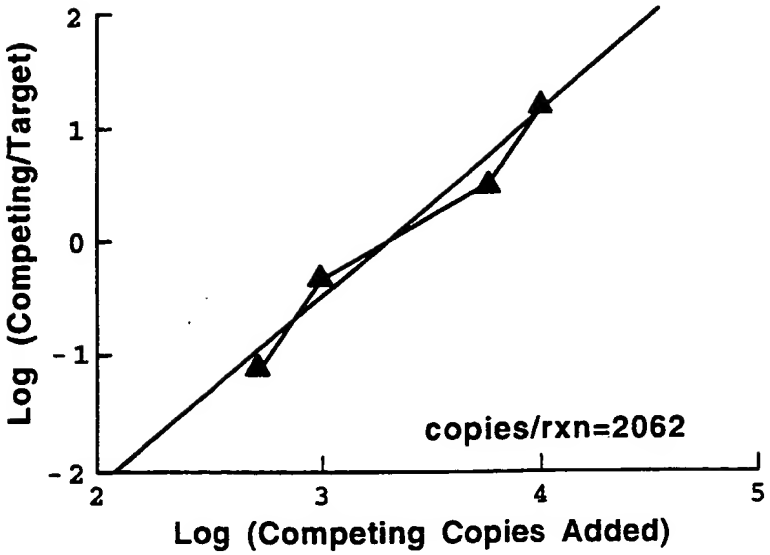

Fig. 2C

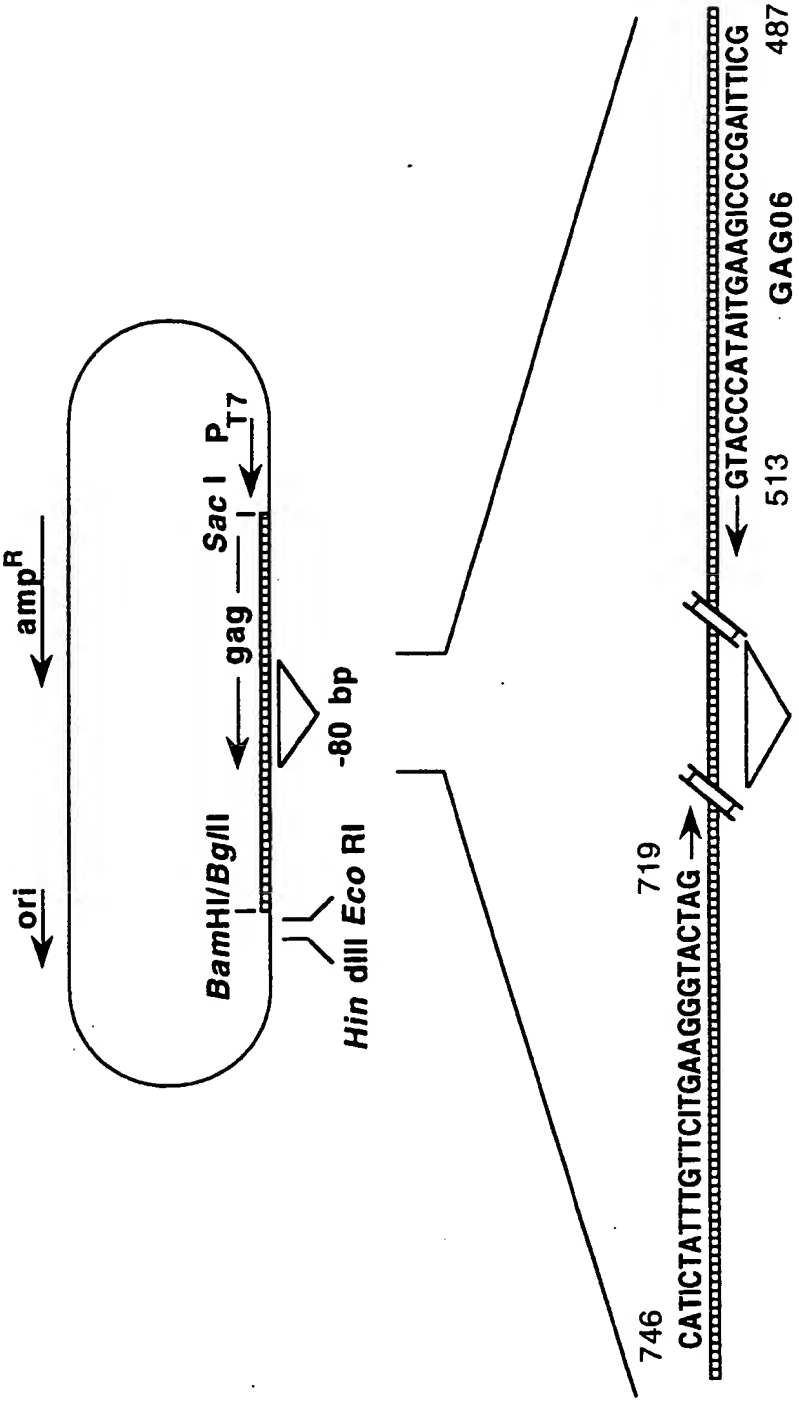
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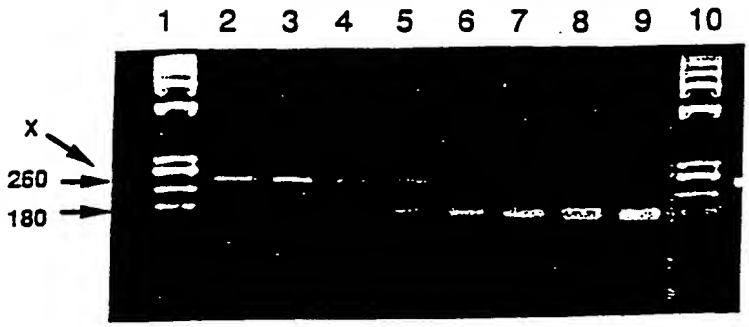
Fig. 2A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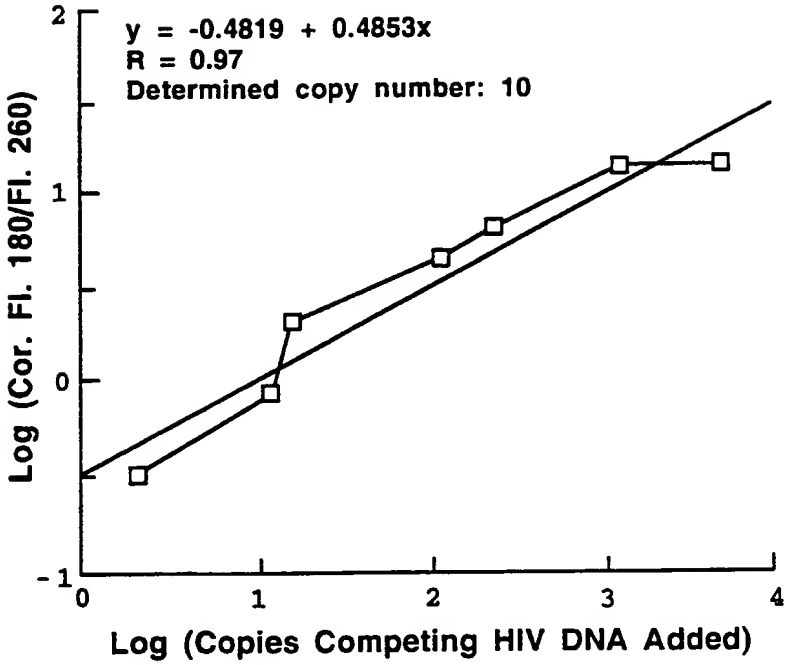
180

Fig. 3A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R = 0.97
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1
2
3
4
Log (Copies Competing HIV DNA Added)

Fig. 3B

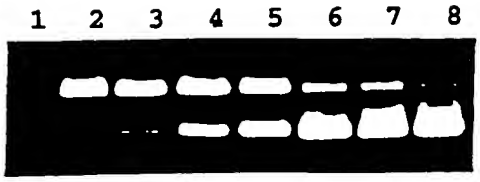
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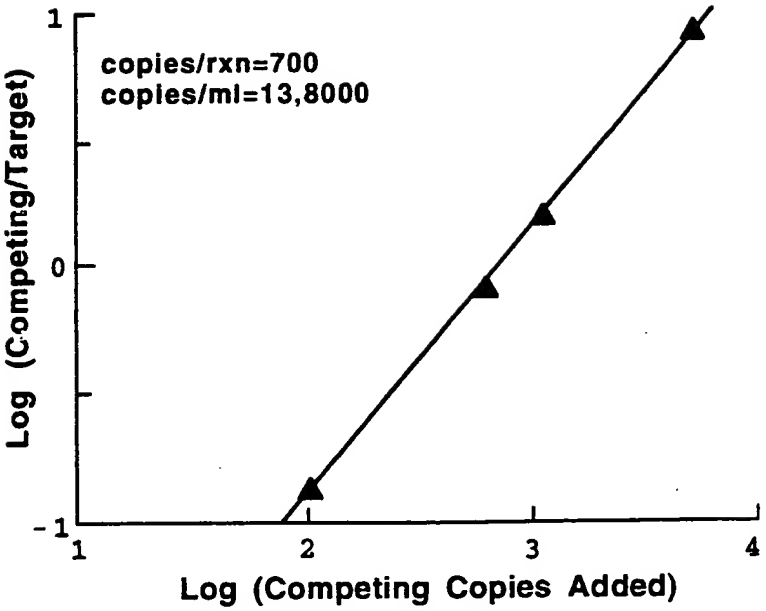
Fig. 4A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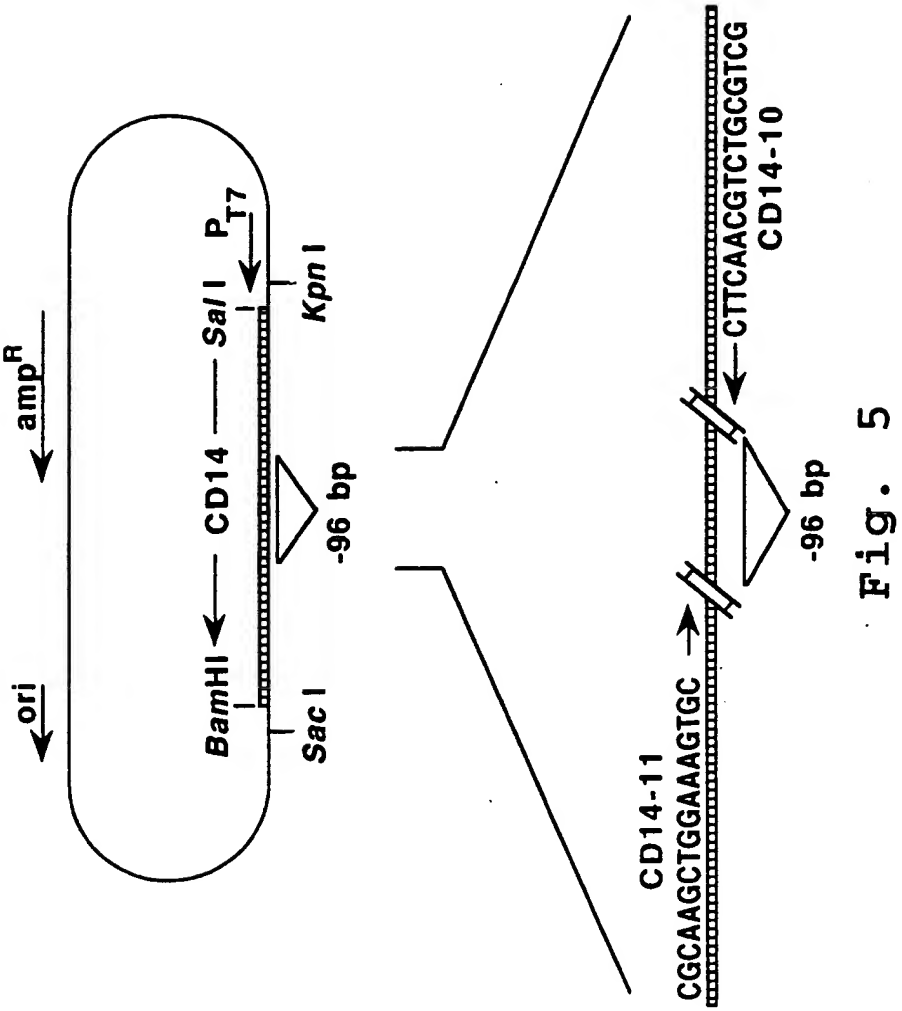
Log (Competing Copies Added)

Fig. 4B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BamHI
CD14
Sal I
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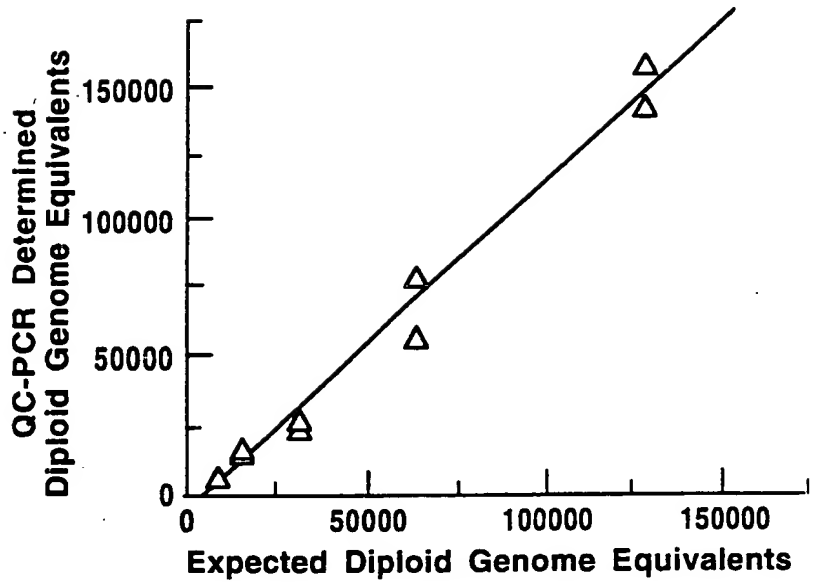
Fig. 5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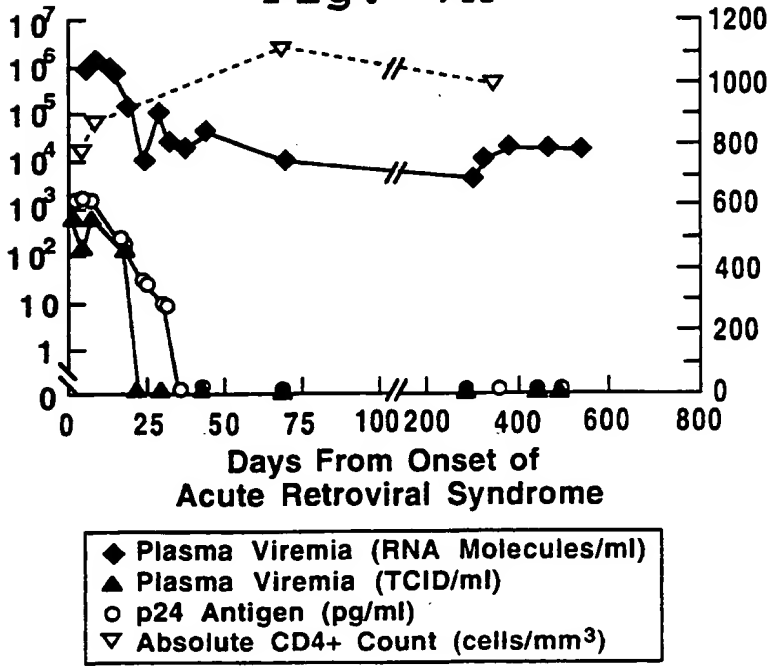
50000
100000
150000
Expected Diploid Genome Equivalents

Fig. 6

Fig. 7A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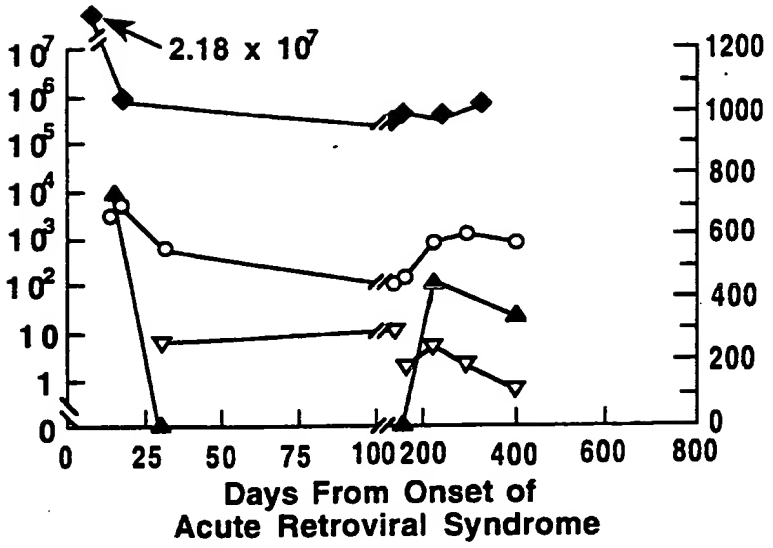
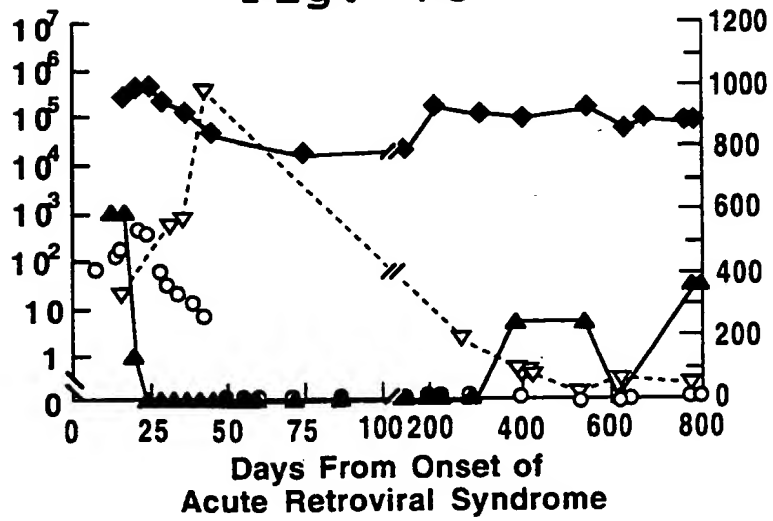
10^6
10^5
10^4
10^3
10^2
10
1
0
1200
1000
800
600
400
200
0
0 25 50 75 100 200 400 600 800
Days From Onset of Acute Retroviral Syndrome
◆ Plasma Viremia (RNA Molecules/ml)
▲ Plasma Viremia (TCID/ml)
o p24 Antigen (pg/ml)
▽ Absolute CD4+ Count (cells/mm3)

Fig. 7B



◆ Plasma Viremia (RNA Molecules/ml)
▲ Plasma Viremia (TCID/ml)
o p24 Antigen (pg/ml)
▽ Absolute CD4+ Count (cells/mm^3)

Fig. 7C



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/02364

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12Q1/68 C12Q1/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOTECHNIQUES vol. 14, no. 1 , January 1993 , EATON PUBL. CO., MA,US; pages 70 - 80 M. PIATAK ET AL. 'Quantitative competitive polymerase chain reaction for accurate quantitation of HIV DNA and RNA species' cited in the application the whole document	1-6,10, 11
Y	---	7-9
Y	ANALYTICAL BIOCHEMISTRY vol. 189 , 1990 , ACADEMIC PRESS, INC., NEW YORK, US; pages 202 - 208 D.E. KELLOGG ET AL. 'Quantitation of HIV-1 proviral DNA relative to cellular DNA by the polymerase chain reaction' the whole document	7-9

	-/--	

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1994	30.06.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Hornig, H

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/02364

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NUCL. ACIDS RES. vol. 16, no. 9 , 11 May 1988 , IRL PRESS, OXFORD, ENGLAND; page 4173 E. FERRERO AND S.M. GOYERT 'Nucleotide sequence of the gene encoding the monocyte differentiation antigen CD14' the whole document ---	7-9
Y	ANTIVIRAL RESEARCH, SUPPL. I vol. 17 , 1992 , ELSEVIER SCIENCE, AMSTERDAM, NL; page 64 M. PIATAK ET AL. 'Accurate quantitation of HIV DNA and RNA sequences using quantitative competitive polymerase chain reaction (QC-PCR)' The 5th international conference on antiviral research, Vancouver, Canada, March 8-13, 1992; abstract no. 39 see abstract ---	1-11
Y	AIDS vol. 6, no. 7 , July 1992 , CURRENT SCIENCE LTD.,LONDON,UK; pages 635 - 641 S. JURRIAANS ET AL. 'HIV-1 viral DNA load in peripheral blood mononuclear cells from seroconverters and long-term infected individuals' cited in the application the whole document ---	1-11
Y	JOURNAL OF INFECTIOUS DISEASES vol. 165, no. 6 , June 1992 , THE UNIVERSITY OF CHICAGO, US; pages 1119 - 1123 D.T. SCADDEN ET AL. 'Quantitation of plasma human immunodficiency virus type 1 RNA by competitive polymerase chain reaction' cited in the application the whole document ---	1-11
Y	J. CLIN. MICROBIOL. vol. 30, no. 7 , July 1992 , AM. SOC. MICROBIOL., WASHINGTON, DC,US; pages 1752 - 1757 S. MENZO ET AL. 'Absolute quantitation of viremia in human immunodeficiency virus infection by competitive reverse transcription and polymerase chain reaction' cited in the application the whole document --- -/--	1-11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/02364

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	SCIENCE vol. 259 , 19 March 1993 , AAAS, WASHINGTON, DC, US; pages 1749 - 1754 M. PIATAK JR. ET AL. 'High levels of HIV-1 in plasma during all stages of infection determined by competitive PCR' the whole document -----	1-6,10, 11

Form PCT/ISA/210 (continuation of second sheet) (July 1992)